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# Global Gene Expression Profiling of Bovine Immature B Cells using Serial Analysis of Gene Expression

John D. Neill [a]; Julia F. Ridpath [a]; Elizabeth Liebler-Tenorio [b]
[a] USDA, ARS, National Animal Disease Center, Virus and Prion Diseases of Livestock Research Unit, Ames, Iowa, USA [b] Friedrich-Loeffler Institute, Federal Research Institute for Animal Health, Jena, Germany

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# GLOBAL GENE EXPRESSION PROFILING OF BOVINE IMMATURE B CELLS USING SERIAL ANALYSIS OF GENE EXPRESSION

**John D. Neill and Julia F. Ridpath**
*Virus and Prion Diseases of Livestock Research Unit, USDA, ARS, National Animal Disease Center, Ames, Iowa, USA*

**Elizabeth Liebler-Tenorio**
*Friedrich-Loeffler Institute, Federal Research Institute for Animal Health, Jena, Germany*

***The Peyer's patches of the small intestine are important sites of antigen processing. The follicles of the Peyer's patches receive the antigen transported by the M cells at the mucosal surface and then play a major role in development of both protective humoral and mucosal immune responses. Serial analysis of gene expression (SAGE) was employed to derive the global gene expression profile of B lymphocytes isolated from the IPPF. Analysis of the SAGE data revealed the identity of genes and the level to which they are expressed by IPPF B lymphocytes. This analysis indicated that they were metabolically active and that the transcripts encoding proteins necessary for a response to antigen presentation were expressed. These transcripts included the B cell receptor components CD76a and b (Ig-alpha and Ig-beta) and accessory c-Src family kinases Lyn and Blk. Furthermore, many of the positive and negative regulators, and accessory proteins that are necessary for B cell antigen receptor signaling were identified. Also present were transcripts encoding interleukins and their cognate receptors. Overall, this SAGE analysis yielded a global picture of gene expression in IPPF B lymphocytes and provides a starting point for the comparison of gene expression in further functional studies.***

***Keywords***: SAGE; Peyer's Patchi; B cell receptor; B cell function

## INTRODUCTION

The Peyer's patches of the jejunum and ileum are major sites of antigen sampling and function as a local barrier to pathogens (1). The patches are in close proximity to dome regions containing M cells, which serve to process the antigen and present it via transepithelial transport to the lymphoid follicles (2). The follicles of the Peyer's patch in sheep (and probably all large ruminants) contain primarily immature B lymphocytes with a small percentage of T lymphocytes (3). The Peyer's

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Address correspondence to John D. Neill, National Animal Disease Center, 2300 Dayton Ave., Ames, IA 50010, USA. Tel: 515-663-7730; Fax: 515-663-7458. E-mail: jneill@nadc.ars.usda.gov.

patches are a primary source of systemic B lymphocytes (1) and appear to be the site of development of antibody repertoire in cattle (4).

Serial analysis of gene expression (SAGE) is a sequence-based, quantitative technology for the rapid analysis of gene expression profiles (5). SAGE has been used successfully to provide information about the transcriptomes of different cell types and tissues (6–8); in the identification and characterization of signaling pathways (9,10), as well as in gene discovery and identification of novel proteins expressed by cancerous cells that can serve as diagnostic markers (11–14). SAGE has been used to examine gene expression differences between bovine GD3.5 + and GD3.5 – γδ T cell populations (15).

The work presented here represents the first step toward characterizing the repertoire of expressed genes in IPPF B lymphocytes isolated from clinically normal calves. These data represent the normal transcriptome of this cell population and will be used in future studies to further investigate the biology of this very important population of B cells.



## MATERIAL AND METHODS

Bovine immature B cells were isolated from IPPF as described previously (16). Here, IPPF were isolated from four clinically normal 15-week Holstein calves. A four to six-inch section of the ileum immediately proximal to the ileal-cecal junction was removed from animals under anesthesia. These ileal sections were immediately placed in ice-cold Earle's balanced salt solution lacking $Ca^{+2}$and $Mg^{+2}$. The digesta was removed and the section opened by cutting along its length. The section was placed mucosa side down in ice-cold Earle's salts, and the serosa was separated from the mucosa using a scalpel blade and gently cutting between the layers. The follicles, appearing as grapelike projections, were scraped into the medium. The follicles from all animals were pooled at this point. The medium containing the follicles, small pieces of mucosa, and individual cells was pipetted into a 50 ml conical tube and the follicles allowed to settle out for 3 to 5 min on ice. The medium was gently aspirated leaving the follicles, and 30 ml of fresh medium was added. This wash was repeated once. The follicles were resuspended in 5 ml of medium and pipetted up and down repeatedly against the side of the tube to disrupt the follicles to produce individual cells. The cells were prefiltered through a 70 μm nylon filter to remove the large aggregates of cells and finally through a 20 μm nylon filter to remove all but single cells. The cells were collected by centrifugation at 400 × g for 10 min at 4°C. The cell population derived from the IPPF was found to be composed of >96% B lymphocytes as determined by immuno-histochemical staining of surface IgM (17). The remainder were primarily T lymphocytes, granulocytes, and ileal epithelial cells. The cell pellet was resuspended in Trizol reagent (Invitrogen, Inc., Carlsbad, CA), and total cellular RNA was isolated using the protocol as provided by the manufacturer. Total poly(A)$^+$ RNA was isolated from this RNA and was used to construct the SAGE library (18). A detailed protocol can be obtained at www.sagenet.org. The sequencing, tag identification, and data analysis were done as previously described (19). The tag sequences were extracted from the raw sequence data using SAGE 2000 version 4.0 software.

**Table 1** Real-time PCR primer sets

| Transcript | PCR primers[1] |
| --- | --- |
| β-actin | CGCCATGGATGATGATATTGC<br>AAGCCGGCCTTGCACAT |
| GAPDH | GCATCGTGGAGGGACTTATGA<br>GGGCCATCCACAGTCTTCTG |
| β-tubulin-1 | TTCAAGCGCATCTCGGAGCAGTT<br>ACACCAGGTCGTTCATGTTGCT |
| COX Vb[2] | TCTGGAGGTGGTGTTCCTACTGAT<br>TCCTTGGTACCTGAGGTTGCCTTT |
| 14-3-3θ | AGACTCGTACAAAGACAGCACCCT<br>TCTGCCGCATCACATTCTTCTCCT |
| TRAM[3] | GTTGCCATGCTGCTAGAAAGTGCT<br>AATGGCAGAGATTTGGTTCCACGG |
| eIF-2α[4] | ACAACCACCCTGGAGAGAACAGAA<br>ATCTGTGACCACTTTGGGCTCCAT |
| BAD | AATGAAGAGACGGAGGAGGAGGAT<br>TTGAAGGAGACGTGAAACTCGTCG |
| eIF-4e[5] | CCCTACTTGATAACATTAGTGATTCTC<br>GTCATATTCCTGGATCCTTCACCA |
| $BclX_l$ | TAGGGTCCCTGAGCATGCTTTCTT<br>AGGAGGGAGGAGACAGGAAATGA |
| sec61α | TTGTTCGGCATGACCATCACCATC<br>CAGAACAATCAGGCCAGCCACAAA |
| PKD2[6] | AGAAGGTGTTCGTGGTGATGGAGA<br>CTGGTTTCAAGTCACAGTGGACGA |
| P58IPK[7] | TCCTCTCCGATCCAGAAATGAGGA<br>TCCGCCTGAGCTAAAGGGATTGAA |
| semaphorin 4B | AGCGCCAGTCCAGAAGAGTC<br>GCAGTTATCAGTGGTGCACG |
| semaphorin 4D | ACGGTCCTATCATCACCGACATCA<br>CTCTTTGCCACGGAAAGAAAGCCT |

[1]top sequence plus sense, bottom sequence minus sense.
[2]cytochrome *c* oxidase Vb.
[3]translocating chain-associating membrane protein.
[4]eukaryotic initiation factor 2α.
[5]eukaryotic initiation factor 4e.
[6]protein kinase D2.
[7]p58 inhibitor of double-stranded RNA-inducible protein kinase (PKR).

To determine the accuracy of the SAGE tag counts for determining expression levels in this study, studies were done to compare real-time PCR with the tag counts. Real-time PCR was done in 20 μl reaction volumes using DyNAmo HS SYBR Green qPCR reaction mix (New England Biolabs, Beverly, MA) according to supplier's specifications. All primers were used at 300 nM final concentration. The primer sequence for all primers used in this analysis are illustrated in Table 1. PCR cycling conditions were 95°C for 15 min, and 40 cycles of 94°C for 10 sec, 60°C for 30 sec, and 72°C for 30 seconds. Analysis of amplification products was done by melt curve where the PCR reactions were heated from 50 to 94°C at a rate of 0.5°C/sec. All real-time PCR analysis was done using an Opticon 2 fluorescent thermocycler

(MJ Research, Waltham, MA). For real-time PCR and SAGE tag count comparisons, β-actin served as the internal control where the number of SAGE tags for β-actin derived from this library and the amplification curve from real-time PCR were considered equal and were the basis for comparison of all genes analyzed. Relative expression levels for the other genes were calculated by the method of Liu and Saint (20).

## RESULTS AND DISCUSSION

The IPPF B cell SAGE library was sequenced to a total of 95,839 tags. This SAGE tag database was submitted to GenBank and has the accession number GSM11383. This level of sequencing of the SAGE library allowed for the detection and identification of transcripts that were expressed at very low levels in IPPF B lymphocytes. For annotation purposes, this tag database was merged with a database of tag-to-gene identifications prepared from the TIGR *Bos taurus* gene index (BtGI) database (www.tigr.org/tdb/tgi/btgi/). A partial list of identified tags with their corresponding mRNA transcripts are shown in Tables 2 and 3. Further, relative expression levels of specific transcripts could be deduced based on frequency of tag sequences in the database.

Table 2 shows the tag counts and transcript identification of the 25 transcripts with the highest level of expression in IPPF B cells. Interestingly, 19 of the 25 were



**Table 2** Twenty-five highest expressed transcripts

| Tag sequence | Count | Transcript |
|---|---|---|
| TAGGTTGTCT | 1877 | translationally controlled tumor protein |
| GGCTTTGGTC | 1312 | acidic ribosomal protein P1 |
| CTGGGAAATT | 1072 | ribosomal protein L27a |
| GCCTGATGGG | 1057 | ribosomal protein L21 |
| GATTCCTAGT | 829 | unknown |
| AAAGAGAGAA | 808 | ribosomal protein S24 |
| GGCTTCGGCT | 649 | acidic ribosomal protein P2 |
| GCAGAGCTTT | 642 | guanine nucleotide-binding protein beta 2 |
| GCCGGCCCGG | 591 | ribosomal protein S15 |
| CGCTGGTTCC | 576 | ribosomal protein L11 |
| ATGTTATTTC | 550 | ribosomal protein L39 |
| ATTCTTTGGT | 550 | ribosomal protein L23 |
| CTCACCAATA | 545 | ribosomal protein L10 |
| AGACAGACAG | 523 | translation elongation factor eEF-1 alpha |
| TTGGTGAAGG | 460 | thymosin beta-4 |
| AACAGGTCCC | 456 | ribosomal protein S25 |
| GAGCCCGCAG | 429 | IgM heavy chain, membrane form |
| TTGGCAAATT | 422 | ribosomal protein L14 |
| TCGGTCTGGG | 410 | ribosomal protein S2 |
| GGCAAGCCCC | 392 | ribosomal protein L10A |
| TCCGTGCACC | 379 | ribosomal protein S11 |
| TAAGGATCCA | 378 | ribosomal protein S26 |
| GCCGAGGAAG | 363 | ribosomal protein S12 |
| AAGAGAACCT | 345 | ribosomal protein L35 |
| GCCTTTAAAG | 335 | ribosomal protein S20 |

**Table 3** Identification and expression level of cellular transcripts encoding proteins important for B cell function

| SAGE tag | Gene description | Tags |
|---|---|---|
| Immunoglobulin | | |
| GAGCCCGCAG | IgM heavy chain membrane form | 429 |
| GACCCCTGAG | immunoglobulin lambda light chain | 221 |
| AGTGCAGACT | IgM heavy chain secretory form | 108 |
| ACGCCTATAC | immunoglobulin kappa light chain | 9 |
| TCAGAGGTGG | IgA heavy chain | 6 |
| TTTTGGAAAT | Mu and alpha Ig-associated J chain | 31 |
| B cell receptor complex | | |
| GCCACTTAGT | B cell receptor α (Ig-α) | 33 |
| TCCGGCTCTC | B cell receptor β (Ig-β) | 4 |
| GGGAGGAGGG | CD19 | 9 |
| TGCATTTCAA | CD21 | 14 |
| GTGCTCTGCT | CD81 | 8 |
| B cell receptor positive effectors | | |
| CCTGTGCAGT | Blk | 4 |
| CGGATTCCCC | Lyn | 10 |
| TGTGGCCTGT | Lck | 5 |
| TACATTTCTA | CD45 | 11 |
| B-cell receptor negative effectors | | |
| CCTCAGCCCG | SHP1 | 4 |
| ACCAATAAAT | SHIP | 13 |
| CGGTACCTGG | CD72 | 2 |
| AGGACCCCGC | Csk | 6 |
| Intracellular signaling proteins | | |
| GTCAGCTGTA | PI3 kinase regulatory subunit | 1 |
| GAAAAGAGAA | PI3 kinase catalytic subunit β | 1 |
| TCTTGTGATA | IKKα | 2 |
| GACTTGTATA | IkB α | 10 |
| AATTTTATTT | signal transduction protein Lnk | 6 |
| ATAGCTGGGG | mitogen activated protein kinase kinase 1 | 6 |
| GCCTCAGGAT | MAPKKK II | 4 |
| CGAATCAAGT | JNK kinase 2 | 3 |
| Adaptor/linker proteins | | |
| TAAAATTATT | Nck | 2 |
| CTTACAGCAA | BANK | 3 |
| CAGCCTTGCA | Grb2 | 6 |
| CATTTCCTCC | Vav1 | 11 |
| CAGGTCAGGG | Vav2 | 4 |
| Cell surface receptors and ligands | | |
| TACAGCACGT | IL 1 type 1 receptor | 1 |
| TATGTTTTCT | IL 2 receptor α subunit | 1 |
| TGCGATATTG | IL 10 receptor β | 1 |
| ATAAACCTAA | CXC chemokine receptor 4 (CXCR4) | 91 |
| ATTTCCTCCA | polymeric immunoglobulin receptor | 16 |
| CTGCTGTAAT | bovine leukemia virus receptor | 10 |
| TTTTTGGTAA | Fas receptor | 5 |
| CATTGGAGAA | IL 1β | 7 |
| CTTGCCAGCT | IL 10 | 2 |
| CATTTGCTTT | CXCL13 | 2 |

(*Continued*)

**Table 3** Continued

| SAGE tag | Gene description | Tags |
|---|---|---|
| TATGTTTTCT | CD25 | 1 |
| ACCTTGAAA | CD44 | 2 |
| TGGCCAGCCC | semaphorin 4B | 1 |
| ATAGGATTTG | semaphorin 4D | 3 |
| Apoptosis | | |
| GTGAAGGGGT | caspase 6 | 16 |
| CTATGGTATC | caspase3 | 2 |
| AAACAACACC | caspase 7 | 2 |
| ATTTGCTTTT | caspase 8 | 1 |
| CTGAACTGTA | caspase 13 | 2 |
| GAGGCACTGG | BAD | 1 |
| CGGCCCCTGT | Bcl-2 | 1 |
| CTTTCTTGCC | Bcl-$X_l$ | 7 |
| CCAGCCCTGT | FADD | 2 |
| ACTGAGGAAG | DAXX | 4 |
| TAGGCAAATT | apoptosis inducing factor (AIF) | 5 |
| TGCAAGGACA | A1 | 9 |

transcripts encoding ribosomal proteins. The transcript encoding translation elongation factor alpha was present with 523 tags. This illustrates the high level of protein translation that takes place in these cells. The transcript with the highest expression level encoded translationally controlled tumor protein and constituted 1.95% of the total mRNA in these cells. In addition, the transcript encoding the membrane form of the IgM heavy chain was expressed at 422 tags out of 95,839 total tags (0.4% of the total mRNA).

Table 3 shows the expression level of genes that are important to B lymphocyte function. These include transcripts encoding immunoglobulin subunits, components of the B cell receptor complex, adaptor molecules, and proteins involved in intracellular signal transduction. All the genes identified in this study were consistent with known B cell physiology and function.

The immunoglobulin gene with the highest level of expression was the membrane form of the IgM heavy chain. Also present were transcripts encoding the IgM secretory form, lambda light chains, and at a very low level, kappa light chains and IgA heavy subunit (108, 221, 9, 6 tags, respectively). These results are in good agreement with those obtained by flow cytometry of surface immunoglobulin expression in purified sheep ileal Peyer's patch follicular cells (16).

Transcripts encoding proteins that comprise the B cell receptor complex were identified (Table 3). These included proteins making up the B cell receptor, Ig-α and Ig-β (CD79a and b), and the co-receptors CD19, CD21, and CD81. Ig-α and Ig-β contain immunoreceptor tyrosine-based activation motifs (ITAMS) that are recognized and phosphorylated by associated members of the Src family of protein kinases, followed by recruitment of downstream Src protein kinases during initiation of B cell receptor signaling (21). The CD79b gene was expressed at a level roughly eightfold lower than the CD79a gene (4 tags vs. 33 tags, respectively) in this population of cells. It is possible that this level of expression of Ig-β is the result of some

Ig-β transcripts possessing alternate 3′ ends that have yet to be identified. The co-receptor molecules were all expressed at near equivalent levels to that of the CD79 molecules.

SAGE tags corresponding to transcripts encoding the Src family kinases Lyn, Blk, and Lck, kinases involved in the positive regulation of B cell signaling, were also identified. These kinases are important in transduction of the signal from the activated B cell receptor following their recruitment to the phosphorylated receptor complex and subsequent phosphorylation of tyrosine residues of their activation domain, probably by Fyn (21). In addition, 11 tags corresponding to transcripts encoding the protein tyrosine phosphatase (PTP) CD45, an activator of the Src kinase family member Lck, were present in the B cell SAGE database. The tags corresponding to other receptor accessory proteins, such as BLNK and the protein kinases Syk and Fyn, were not possible to identify due to lack of transcript 3′ sequence data available in the bovine EST database.

Tags were also identified that corresponded to expression of negative regulators of B cell signal transduction. These included the PTPs SHP1 and SHIP, as well as the negative regulator of Src family kinases, c-Src kinase (Csk). The PTPs function to maintain specific target molecules in an unphosphorylated, inactive state. The biochemical role of Csk is to negatively regulate Src kinase family members by phosphorylation of a highly conserved tyrosine residue found within the negative regulatory domain in the C-terminus of the kinase molecule. Identification of tags indicating the expression of proteins that act to localize Csk to the plasma membrane of the B cells, such as PAG/Cbl, was not made.

Activation of B cells following stimulation of the B cell receptor requires transfer of the signal from the cell surface to the cytosol, allowing further stimulation of kinase pathways. This function is carried out in part by a number of adaptor/linker proteins. Tags derived from transcripts encoding Grb2, NCK, BANK were present in this SAGE library at 6, 2, and 3 tags, respectively. This illustrates their relatively low levels of expression. Tags corresponding to the adaptor proteins BLNK, SOS, and SHC1 were not possible to identify, again because of lack of 3′ transcript sequence data in the bovine EST database.

Tags derived from transcripts encoding protein kinases involved in other intracellular signaling pathways not related to the B cell receptor, such as PI3 kinase regulatory and catalytic subunits, MAPKK, MAPKKK II, JNK kinase 2, and Bruton's tyrosine kinase (BTK), were present, indicating that these pathways are intact in these cells. Activated PI3 kinase has AKT (tag not found) and BKT as substrates. Activated BKT can then phosphorylate IKKα resulting in degradation of IκBα. This allows transport of the transcription factor NFκB to the nucleus, an event observed following stimulation of the B cell receptor (22). Tags derived from the IKKα and IκBα transcripts were present at 2 and 10 tags, respectively.

Tags corresponding to the expression of genes encoding IL-1β, IL-2, and IL-10 as well as the interleukin receptors IL1 type 1, IL2 alpha subunit, and IL10β and the Fas receptor were identified. The activation markers CD25, CD44, and CD62, an adhesion molecule shed from activated cells, were present at very low levels. Tags derived from the chemokine receptor CXCR4 transcripts were found in the database, albeit at very low levels. CXCR4 is necessary for the normal production of B lymphocytes (23). The transcript encoding the ligand of the CXCR5 chemokine receptor, CXCL13, was identified. The CXCL13 ligand plays a role in the homing of B lymphocytes to the Peyer's patch (24). The tag corresponding to the CXCR5 receptor transcript was not found in the database. These genes, with the exception of CXCR4, were expressed at very low levels, ranging from 0.001% to 0.034% of total mRNA.

Proteins comprising the apoptosis pathways are necessary for proper regulation of the immune response by causing death of cells no longer necessary or those recognizing self-antigens. The majority of B cells of the ileal Peyer's patch ($\geq$95%) are killed by apoptosis (25,26), indicating an intact and very efficient means of apoptotic induction. Transcripts encoding caspases 3, 7, 8, and 13 were present at very low levels of approximately 0.001 to 0.002% of the total. In contrast to this, caspase 6 was apparently transcribed at a high level as indicated by 16 tags present in the database with a calculated expression level of 0.017%. Also present were transcripts encoding the anti-apoptotic proteins A1, Bcl-2, and Bcl-$X_l$. The adaptor proteins FADD and DAXX, proapoptotic protein BAD, and apoptosis-inducing factor were also identified. These results demonstrate that all components of the apoptotic pathways are present and are required at very low levels of expression in these cells.

Another use of SAGE data is in gene discovery. In the case of IPPF B cells, this was the identification of gene expression not previously known to take place in these cells. An example was the identification of the tag sequence derived from the transcript encoding semaphorin 4B. The semaphorins are a family of proteins, found primarily in the central nervous system, that regulate or guide the growth of neurons (27,28). In the immune system, semaphorin 4D (CD100) is highly characterized and plays a role in activation of dendritic and B cells by T cells (29,30). The presence of semaphorin 4B transcripts in IPPF B cells was confirmed by PCR (data not shown) and by real-time PCR (Table 4). The expression of this gene has never been described

**Table 4** Comparison of SAGE tag counts and real-time PCR for specific transcripts

| Transcript | SAGE tags | rt-PCR predicted tags |
|---|---|---|
| β-actin[1] | 70 | 70 |
| GAPDH | 41 | 47.8 |
| β-tubulin 1 | 24 | 27.7 |
| COX Vb[2] | 32 | 27.5 |
| 14-3-3 θ | 14 | 17.5 |
| TRAM | 10 | 9.8 |
| eIF2α | 11 | 10.6 |
| BAD | 6 | 4.7 |
| eIF-4e | 7 | 10.5 |
| Bcl-$X_l$ | 7 | 5.8 |
| sec61α | 4 | 6.8 |
| PKD2[3] | 2 | 3.8 |
| P58IPK[4] | 4 | 5.5 |
| semaphorin 4B | 1 | 1.5 |
| semaphorin 4D | 4 | 1 |

[1]β-actin internal control.
[2]cytochrome c oxidase Vb.
[3]protein kinase D2.
[4]p58 inhibitor of double-stranded RNA-inducible protein kinase (PKR).

in B cells, and the function of the protein is unknown. However, semaphorin 4B is believed to be expressed on the cell surface and may function as a receptor in cell-to-cell interactions (31).

The results of the real-time PCR/SAGE tag count analysis of gene expression levels in IPPF B cells are shown in Table 4. Transcripts with a range of expression levels as indicated by SAGE were included to determine if different expression levels would affect the ability to predict SAGE tag count based on real-time PCR results. Based on this analysis, a high degree of confidence can be placed on the SAGE data as an indicator of transcript number in IPPF B cells. There was a high degree of correlation ($r^2 = 0.98$) between the real-time PCR and the SAGE library for level of expression for the genes examined. This analysis provides support that the SAGE tag counts reflect the actual levels of gene expression in these B cells.

The gene expression profile of ileal Peyer's patch–derived B lymphocytes from clinically normal calves generated in this study provides a strong starting point for a number of analyses of the function of these cells. Results obtained in this study are in strong agreement with what is known about B cell physiology and function. In addition, there was a considerable amount of data that was not presented here that provides insight into metabolic state, expression of other cell surface markers and receptors, and other signaling pathways. Transcripts were found for most of the proteins with major roles in B cell receptor signaling or in regulation or moderation of this signaling process. Additional sequencing of this SAGE library will most likely show the presence of other important proteins that are expressed at even lower levels. As indicated above, the major obstacle to the identification of bovine SAGE tags is the amount, as well as the position (5′ vs. 3′), of the sequence data available in the bovine EST databases. However, with recent increased sequencing of bovine ESTs from the 3′ end, the ability to identify bovine SAGE tags has increased. This is evident with the identification of transcripts that are present in these cells at very low levels. In addition, this SAGE data can be used in annotation of the bovine genome as sequence becomes available, much as has been described for other genome projects (32,33).



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